The culturable mycobiota of a Mediterranean marine site after an oil spill: isolation, identification and potential application in bioremediation

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Abstract

Bioremediation of marine environment could be the response to oil spills threats. In the present study the fungal community from a Mediterranean marine site chronically interested by oil spills was investigated. Sixty-seven taxa were isolated from water sample and 17 from sediments; for many of the identified species is the first report in seawater and sediments, respectively. The growth of 25 % of the fungal isolates was stimulated by crude oil as sole carbon source. Four strains were selected to screen hydrocarbons degradation using the 2,6-dichlorophenol indophenol (DCPIP) colorimetric assay. *A. terreus* MUT 271, *T. harzianum* MUT 290 and *P. citreonigrum* MUT 267 displayed a high decolorization percentage (DP ≥ 68 %). *A. terreus* displayed also the highest decreases of hydrocarbons compounds (up to 40 %) quantified by gas-chromatography analysis.

These results suggest that the selected fungi could represent potential bioremediation agents with strong crude oil degradative capabilities.

Keywords: marine fungi; crude oil; biodegradation; Mediterranean Sea; *Aspergillus terreus*

1. Introduction

Most of oil worldwide production is transported by sea, therefore costal and marine environments are strongly exposed to accidentally oil spills (McGenity et al., 2012; U.S. Energy Information Administration, EIA, 2014). The effects of such events are incalculable, influencing human life style, the cost environment and the whole wildlife. For instance, after the Prestige accident in 2002 the most affected beaches lost up to 66 % of species richness (De la Huz et al., 2005); the Deepwater Horizon spill (2010) compromised biodiversity of vertebrates (Antonio et al., 2011) and metazoan meiofauna (Baguley et al., 2015).

Once released in the environment, oil prevents light diffusion and oxygen penetration. Its carcinogenicity and mutagenicity has been already demonstrated, driving also the U.S. EPA to list

16 Polycyclic Aromatic Hydrocarbons (PAHs) usually present in crude oil mixture as priority pollutants (Balachandran et al., 2012; Matsubara et al., 2006).

Nowadays crude oil removal is possible using different techniques, such as *in situ* burning or the use of chemical dispersants, but they have economic, ecologic and technic drawbacks: for instance the use of dispersant does not solve the problem but, just separate oil in another phase which cannot easily be removed from the environment (Schaum et al., 2010; Zheng et al., 2014). Bio-based systems offer interesting alternatives being an economic and environmental friendly method (Zhang et al., 2011). The use of bacteria and fungi is based on their capability to use crude oil and its derivatives as the sole carbon source (McGenity et al., 2012). Their adaptation skills and robustness to survive in extreme environments could lead them to be agents of bioremediation process. Bioremediation of crude oil spill is a well-known practice to treat polluted terrestrial environments (Marco-Urrea et al., 2015), but in marine ecosystems the knowledge is still rough, and its primarily focused on prokaryotic organisms. Harayama and collaborators (2004) demonstrated that bacteria such as *Alcanivorax* spp. and *Cycloclasticus* spp. are able to use aliphatic and aromatic hydrocarbons, respectively. Many bacteria can also produce biosurfactants, which are very important in order to reduce the surface tension and increase the uptake of crude oil (Das and Chandran, 2011; Yakimov et al., 1998). Nevertheless some factors could affect their efficiency as nutrient scarcity (i.e. C:N:P ratio) (Crisafi et al., 2016; Hassanshahian and Cappello 2013; Jimenez et al., 2007) and crude oil composition, being saturated hydrocarbons most biodegraded than aromatic ones (Wang et al., 2013).

There are few evidences indicating that under some circumstances, fungi could be better degraders of petroleum than traditional bioremediation techniques recruiting bacteria (Saravanan and Sivakumar, 2013). Actually, fungi have been recently reported for their capability to use PAHs as benzo[a]pyrene (Passarini et al., 2011) and to produce biosurfactants (Kirianet al., 2009). Unfortunately the knowledge of the fungal community associated to marine contaminated sites is scarce, being the central topic of just few researches (Al-Nasrawi, 2012; Elshafie et al., 2007; Sadaba and Sarinas, 2010; Salmanov et al., 2008; Salvo et al., 2010; Simister et al., 2015).

The aim of this work was to isolate and to identify the fungal community of an Italian marine site contaminated by an oil spill, in order to better understand the role of fungi in marine crude oil contaminated environments. The capability of the isolated fungi to use crude oil as sole carbon source was also assessed.

2 Materials and Methods

2.1 Study area and sampling survey

Samples were collected from a marine site chronically and recently contaminated by oil spill in Gela, Sicily (37°03'31.58" N; 14°15'27.32" E). The last episode took place on June 4, 2013, when more than 13.500 L of heavy crude oil entered into the sea. Three days after the oil spill, 1.5 L of seawater at one-meter depth and about 200 g of sediments near the shorelines were collected, stored at 4 °C and transported to the laboratory. Main physical-chemical parameters (pH, temperature, salinity, redox potential) were measured using a multiparameter probe Waterproof CyberScan PCD 650 (Eutech Instruments, The Netherlands). Determination of oxygen concentration at chosen depths was carried out using the Winkler method (Carpenter, 1965) with an automatic endpoint detection burette 716 DNS Titrino (Metrohm AG, Herisau, Switzerland). Sediments and water parameters are listed in Table 1.

Table 1. Water and sediments chemical-physical parameters.

	Redox potential (mV)	**Dissolved oxygen (mg L^{-1})**	**Salinity (‰)**	**pH**
Water	50	6.99	36	7.5
Sediments	-140	3.80	32	7.7

2.2 Fungal isolation and identification

One hundred aliquots of 10 mL of seawater sample were filtered through sterile nitrocellulose membranes (50 mm diameter, 0.45 µm pore, VWR); the membranes were transferred onto 9 cm diameter Petri dishes containing 20 mL of Corn Meal Agar Sea Salt medium (CMASS: 2 g corn meal, 20 g Sigma sea salt mix, 15 g agar, 1 L H_2O) added with antibiotics (streptomycin 0.015 g L^{-1}, chloramphenicol 0.05 g L^{-1}). Plates were incubated at 24°C and monitored daily for one month to allow the isolation of slow-growing colonies. Each strain was isolated in pure culture for the taxonomic identification. The number of colony forming units (CFU) per 100 mL of seawater (CFU 100 mL^{-1}) was calculated both for the total mycoflora and for each species or morphotype. The relative abundance (RA) was estimated as CFU for each species out of the total CFU. Moreover, 10 sterile cottons swabs were scraped on the inner wall of the bottle containing the sample (where a visual analysis highlighted the presence of mycelium onto oil droplets) and then plated onto 9 cm diameter Petri dishes containing CMASS and antibiotics.

As regard sediment, the soil dilution plate technique was applied: the equivalent of 20 g dry weight was suspended in 200 mL phosphate buffer solution 0,2 M, pH 7. The final dilution (1:100) was plated (1 mL per plate) onto 20 Petri dishes (15 cm diameter) containing 30 mL of CMASS plus antibiotics. Plates were incubated in the dark at 24°C and treated as described above. The number of

CFU per g of dry weight (CFU g^{-1} dw) was calculated both for the total mycoflora and for each species or morphotype.

Fungi were classified according to their macroscopic and microscopic features and by molecular analysis. Genomic DNA was extracted from mycelium, following the protocol of Graham and collaborators (1994), and a Polymerase Chain Reaction (PCR) was performed. For the genera *Cladosporium* and *Alternaria*, the actin gene (Bensch et al., 2012) and the glyceraldehyde-3-phosphate dehydrogenase gene (Berbee et al., 1999) were amplified, respectively. For both *Penicillium* and *Aspergillus* genera the β-tubulin gene was amplified (Glass e Donaldson 1995). For all the other genera and sterile mycelia the ITS1-5,8S-ITS2 region was used for PCR amplification (White et al., 1990). Strains were deposited at the *Mycotheca Universitatis Taurinensis* (MUT) of the University of Turin. Sequences were registered in GenBank database.

2.3 Direct observation of fungi in water and sediment samples

The fluorochrome Fluorescent Brightener 28 (FB, Sigma-Aldrich) was used in order to evaluate the presence of actively growing fungi in seawater and sediment samples. One mL of FB was added to 20 mL of seawater and 2 g of sediment dissolved in 20 mL of sterile water. The samples were incubated at 24 °C in the dark for two hours. Each sample was filtered onto sterile Anodisc membranes (0.2 µm pores Sigma) that have been observed under an epifluorescence microscope (Leica DFC 320) with UV illumination (365 nm) to detect fungal hyphae and spores (Alef and Nannipieri, 1995).

2.4 Fungal growth on crude oil

All isolated fungi were tested for their capabilities to grow on crude Arabian Light oil as sole carbon source. Fungi were pre-grown in 9 cm Petri dishes onto the mineral medium ONR7a (Dyksterhouse et al., 1995) added with 2 g L^{-1} of malt extract and incubated at 24 °C in the dark for 7 days. Mycelium disks (4 mm diameter) were taken from the margin of the actively growing colonies, and inoculated in 9 cm plates containing ONR7a with (1:10 w/v of Arab Light oil in hexane) and without crude oil (control). Plates were inoculated in triplicates and incubated in the dark at 24 °C and the mycelium growth were evaluated after 4, 7 and 14 days. The percentage of stimulation (PS) by crude oil was calculated as follow:

(PS)= - [(average diameter in control plates (mm) - average diameter in crude oil plates (mm)) / average diameter in control plates (mm)] * 100.

Data were analyzed with ANOVA ($p \leq 0.05$) using IBM SPSS Statistics software.

2.5 Crude oil degradation assay

Four strains were selected from the previous experiment and tested for their capability to degrade oil in liquid cultures. The utilization/degradation of crude oil was assessed according to the

modified protocol of Varjani and Upasani (2013) using the 2,6-dichlorophenol indophenol (DCPIP) colorimetric assay. Fungi were inoculated as five agar plugs (4 mm diameter), taken from the margin of an actively growing colony on ONR7a added with 1 % malt extract in 100 mL Erlenmeyer flasks containing 40 mL of liquid ONR7a, 1 % w/v crude oil (1:5 in hexane), 0.016 % w/v DCPIP and 0.1 % Tween 80. Two biotic controls were carried out: i) in presence of DCPIP alone to evaluate their capability to use it for the primary metabolism or to physiologically express oxidative enzymes without the stimulation of crude oil; ii) in ONR7a medium to evaluate the fungal growth without crude oil. Abiotic controls (DCPIP with or without crude oil) were carried out to evaluate possible photobleaching phenomena. The experiment was performed in triplicate.

Flasks were incubated in agitated conditions (110 rpm) at 24 °C in the dark for 10 days. Aliquots of solution were sampled at the beginning of the experiment and after 1, 3, 4, 7 and 10 days. For each sample the UV–Vis spectroscopy measurements (Tecan Infinite M200) were performed at 600 nm (Varjani and Upasani, 2013) and the pH was measured.

At the end of the experiment, the biomasses were filtered and dried at 60 °C for 24 h in order to assess the fungal development by the biomass dry weight.

The collected data were analyzed with ANOVA ($p \leq 0.05$) using IBM SPSS Statistics software.

2.6 Hydrocarbons analyses

Efficiency of petroleum degradation was estimated by the analysis of total extracted and resolved hydrocarbons (TERHC). At the fixed time points, TERCH were extracted from water sample following the analytical method hexane extraction - GC/FID in seawater. Briefly, equal volume of *n*-hexane was added to 40 mL of culture and was further shaken at 150 rpm for 30 minutes, and the extract was passed through a glass column filled with anhydrous Na_2SO_4 (Sigma-Aldrich, Milan). Same treatment was repeated and the extracts were combined and evaporated to complete dryness. Residues were re-suspended in 1 mL of *n*-hexane prior the gas chromatography (GC) analysis (Rocchetti et al., 2011). All measures were performed using a Master GC DANI Instruments (Contone Switzerland), equipped with Split/SplitLess injector (SSL) and Flame Ionization Detector (FID). Sample (1 µL) was injected in splitless mode at 330 °C, temperature program: 50°C (held 4 min), to 155°C at 7°C/min, to 325°C at 6°C/min (held 8 min), to 330°C at 6°C/min (held 1.5 min). The analytical column was a Restek Rxi-5 Sil MS with Integra-Guard, 30 m x 0.25 mm (ID x 0.25 · m film thickness). Helium carrier gas was maintained at a constant flow of 1.5 mL min^{-1}. Data obtained were normalized by 2-ethylphenatrene (internal spike/control of biodegradation) and were used to calculate the relative removal of hydrocarbons.

The collected data were analyzed with ANOVA ($p \leq 0.05$) using IBM SPSS Statistics software.

3 Results

Fungal hyphae and spores were directly detected by staining with FB both in water and sediment samples as shown in Figure 1.

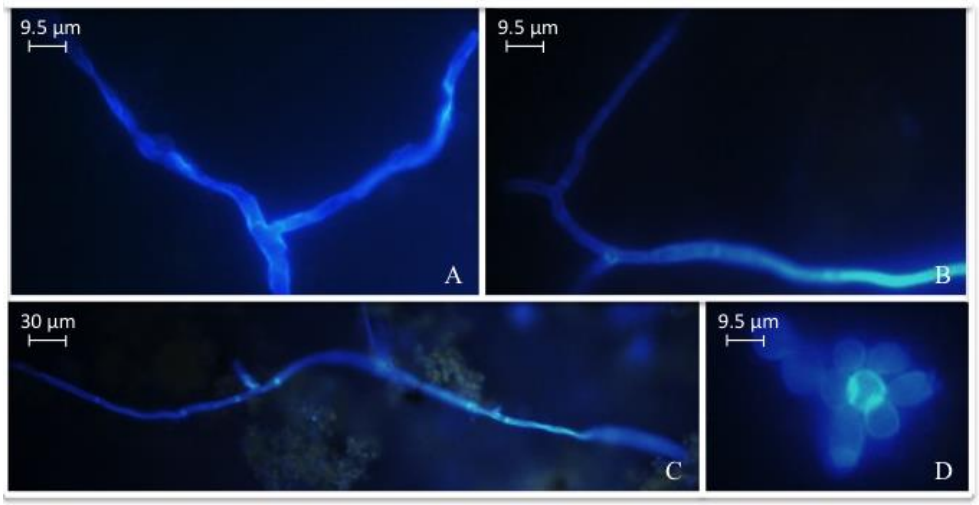

Figure 1. Fungal structure detected by FB at epifluorescence microscope: A) hyphae in water sample; B) and C) hyphae in sediments; D) conidia in sediment.

Table 2 shows the fungal entities isolated from both polluted seawater and sediments, their fungal load and RA (%) and the species first reported in the sampled environment. The water sample showed a total fungal load of about 30 CFU 100 mL^{-1} and a high fungal biodiversity: the cultivable mycoflora consisted of 67 taxa belonging to 29 genera. Almost all the recorded taxa belonged to Ascomycota (94 %). To the best of our knowledge, 12 species had not previously been recorded in a marine environment.

Synchephalastrum racemosum resulted the most abundant species (RA = 23.5 %), followed by *Alternaria tenuissima* and *Chaetomium globosum* (RA > 5 %). Overall, *Penicillium*, *Aspergillus* and *Trichoderma* were the most represented genera with 11, 10 and 5 taxa, respectively.

The use of swabs allowed the isolation of five taxa not retrieved from water filtration. All these fungi, except *Periconia byssoides*, resulted sterile mycelia and were identified only by molecular analyses.

As regards sediments, the total fungal load was about 4,000 CFU g^{-1} dw, and the cultivable mycoflora consisted of 17 taxa belonging to 12 genera (Table 2). Ascomycota predominated (94 %) over Basidiomycota (6 %). To the best of our knowledge, most of the isolated species (14) had not previously been recorded from any marine sediment.

C. cladosporioides resulted the most abundant species (RA = 27.8 %), followed by *Phomopsis ambigua* (RA = 16.7 %); these two taxa represent about half of the total fungal load detected in

sediments. One third of fungi isolated from sediments were sterile in culture despite several attempts to induce sporulation and only 6 species were common to both water and sediments (Table 2).

Table 2. Fungal taxa from water and sediments, fungal load and relative abundance (RA) are reported. Common species of both samples are in bold.

Fungal species in water	**(CFU 100 mL^{-1})**	**RA (%)**	**Fungal species in Sediments**	**(CFU g^{-1}dw)**	**RA (%)**
Syncephalastrum racemosum	7.0	23.5	***Cladosporium cladosporioides*•**	1108.6	27.8
*Alternaria tenuissima***	2.6	8.8	*Phomopsis ambigua*•*	665.2	16.7
Chaetomium globosum	1.7	5.6	*Epicoccum nigrum*•*	277.2	6.9
*Trichoderma harzianum***	1.3	4.3	*Fusarium brachygibbosum*•*	277.2	6.9
Botrytis cinerea	1.1	3.8	*Lecanicillium* sp.*	277.2	6.9
Fusarium solani	1.1	3.6	*Phomopsis* sp.*	277.2	6.9
Stemphylium solani◆	1.0	3.4	*Acremonium sclerotigenum*•*	277.2	6.9
Paradendryphiella arenariae	0.9	3.0	***Cladosporium pseudocladosporioides*•**	277.2	6.9
Penicillium citrinum	0.9	2.9	***Aspergillus calidoustus*•**	277.2	6.9
Trichoderma longibrachiatum	0.8	2.7	*Penicillium solitum*•*	55.4	1.4
Cladosporium pseudocladosporioides	0.7	2.5	***Arthrinium phaeospermum*•**	55.4	1.4
Fusarium sporotrichioides◆	0.7	2.2	*Penicillium olsonii*•*	27.7	0.7
Aspergillus versicolor	0.5	1.8	*Peniophora* sp.*	27.7	0.7
Fusarium oxysporum	0.5	1.6	*Phaeosphaeria avenaria*•*	27.7	0.7
Penicillium antarcticum	0.5	1.6	*Thielavia coactilis*•*	27.7	0.7
Alternaria botryospora◆	0.4	1.3	***Cladosporium bruhnei*•**	27.7	0.7
Rhizopus stolonifer	0.4	1.3	***Penicillium brevicompactum*•**	27.7	0.7
Arthrinium arundinis	0.3	1.1			
Arthrinium phaeospermum	0.3	1.1			
***Aspergillus calidoustus*◆**	0.3	1.1			
Cladosporium cladosporioides	0.3	1.1			
Cladosporium sp.	0.3	1.1			
Curvularia inaequalis◆	0.3	1.1			
Fusarium sp.	0.3	1.1			
Lulworthiales sp. (1)*	0.3	1.1			
Lulworthiales sp. (2)*	0.3	1.1			
Penicillium bialowiezense	0.3	1.1			
Penicillium chrysogenum	0.3	1.1			
Scopulariopsis brevicaulis	0.3	1.1			
Stachybotrys chlorohalonata	0.3	1.1			
Trichoderma koningii	0.3	1.1			
Aspergillus tubingensis	0.3	0.9			
Dictyoarthrinium sacchari◆	0.2	0.8			
Pleospora sp.	0.2	0.7			
Penicillium canescens	0.2	0.6			
Aspergillus niger	0.1	0.4			
Aspergillus sydowii	0.1	0.4			
Aspergillus ustus	0.1	0.4			

Gibellulopsis nigrescens	0.1	0.4
Sedecimiella taiwanensis	0.1	0.4
Chaetomidium fimeti◆	0.1	0.3
Humicola sp.	0.1	0.3
Phoma labilis◆	0.1	0.3
Arthrinium sp.	0.1	0.2
Aspergillus fumigatus	0.1	0.2
Aspergillus insuetus	0.1	0.2
Aspergillus terreus	0.1	0.2
Aspergillus westerdijkiae	0.1	0.2
Cladosporium bruhnei	0.1	0.2
Cladosporium halotolerans	0.1	0.2
Dicyma sp.	0.1	0.2
Emericella nidulans	0.1	0.2
Penicillium brevicompactum	0.1	0.2
Penicillium citreonigrum	0.1	0.2
Penicillium desertorum◆	0.1	0.2
Penicillium selandiae◆	0.1	0.2
Penicillium sp.	0.1	0.2
Penicillium vinaceum	0.1	0.2
Phoma sp.*	0.1	0.2
Stachybotrys chartarum	0.1	0.2
Trichoderma brevicompactum◆	0.1	0.2
Trichoderma virens	0.1	0.2
Corollospora portsaidica	from swabs	
Corollospora sp.*	from swabs	
Peniophora sp.*	from swabs	
Periconia byssoides	from swabs	
*Tyromyces fissilis**◆	from swabs	

*Sterile mycelia in culture; **Isolated by filtration and swabs; ◆first report in marine environments; •first report in beach sediments.

3.2 Fungal growths on crude oil

All the 142 isolated fungi were tested for their capability to grow in presence of crude oil as sole carbon source. All of them were able to grow on it, but with different efficiency compared to controls. At the end of the experiment, 24 % of the 115 water isolates were significantly stimulated, 49 % were insensitive and 27 % were inhibited by the presence of crude oil (Figure 2A). As regard sediment, 22 % of the 27 fungi, were stimulated, 45 % were insensitive and 33 % were inhibited by the presence of crude oil (Figure 2B).

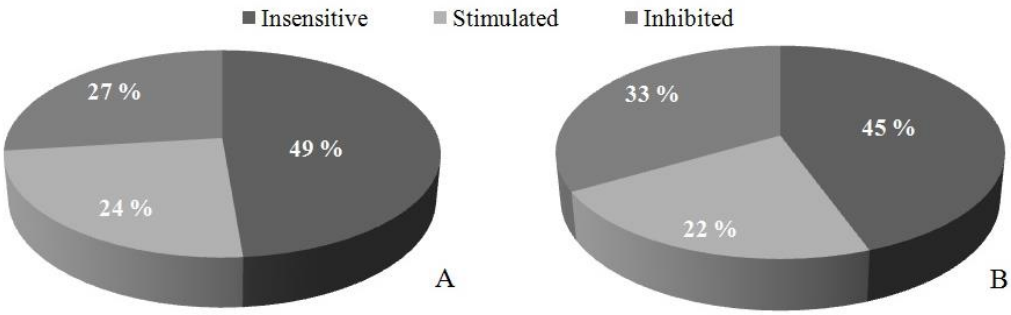

Figure 2. Percentage of fungi from water (A) and sediment (B) insensitive, stimulated and inhibited by the presence of crude oil at 14th day.

The 14 strains that displayed the highest percentages of stimulation (PS ≥ 20 %) were all isolated from water and are listed in Table 3. The highest stimulation was observed for Lulworthiales sp. MUT 263, *P. citreonigrum* MUT 267, *A. terreus* MUT 271 and *T. harzianum* MUT 290 whose radial growths were enhanced in a range between 197 % and 57 %.

On the other hand, several strains belonging to different species i.e. *C. pseudocladosporioides*, *A. tenuissima*, *A. phaeospermum* and *A. tubingensis* were strongly inhibited (up to – 40 %) by crude oil presence (data not shown).

The different behavior of strains belonging to the same species highlighted a marked intraspecific variability (data not shown). For instance *P. citrinum* showed two insensitive and two stimulated strains by the presence of crude oil. As regard *S. racemosum*, 9 strains were inhibited, whereas one was insensitive and one stimulated by crude oil.

Table 3. List of fungal strains that showed the highest percentage of stimulation (PS ≥ 20 %): growth (mm) in presence and without (control) crude oil.

Fungal strains	**Crude oil (mm)**	**Control (mm)**	**PS (%)**
Lulworthiales sp. (1) MUT 263	55	18	197
P. citreonigrum MUT 267	27	14	96
A. terreus MUT 271	86	48	79
T. harzianum MUT 290	81	52	57
A. versicolor MUT 1650	35	25	42
T. harzianum MUT 1832	86	61	41
A. versicolor MUT 1636	41	31	34
P. citrinum MUT 1768	33	26	29

T. harzianum MUT 1851	86	67	28
Penicillium sp. MUT 1779	38	30	27
T. longibrachiatum MUT 1841	86	68	26
C. globosum MUT 284	86	69	24
P. canescens MUT 1815	30	25	22
S. solani MUT 1754	86	71	20

3.3 Crude oil degradation assay

The four strains that displayed the highest PS in the previous experiment were investigated for their capability to degrade crude oil in liquid culture. The ability to use crude oil was evaluated through three parameters: i) the change in color of culture media, from blue to colorless; ii) the growth of fungal biomass; iii) the disappearance of crude oil from the medium. The DCPIP decolorization is illustrated in Figure 3: a fast transformation was observed for *A. terreus* MUT 271 that displayed the highest DP (88 %). Also *P. citreonigrum* MUT 267 and *T. harzianum* MUT 290 activated a strong oxido-reductase cascade able to oxidize about 70 % of DCPIP. Lulworthiales sp. MUT 263 was less effective and much slower in decolorization (23 % at day 10).

The correlation between DCPIP disappearance and crude oil degradation was confirmed by the fact that no decolorization was observed when the crude oil was not added to the solution, indicating that the fungi expressed enzymes only as response to the crude oil.

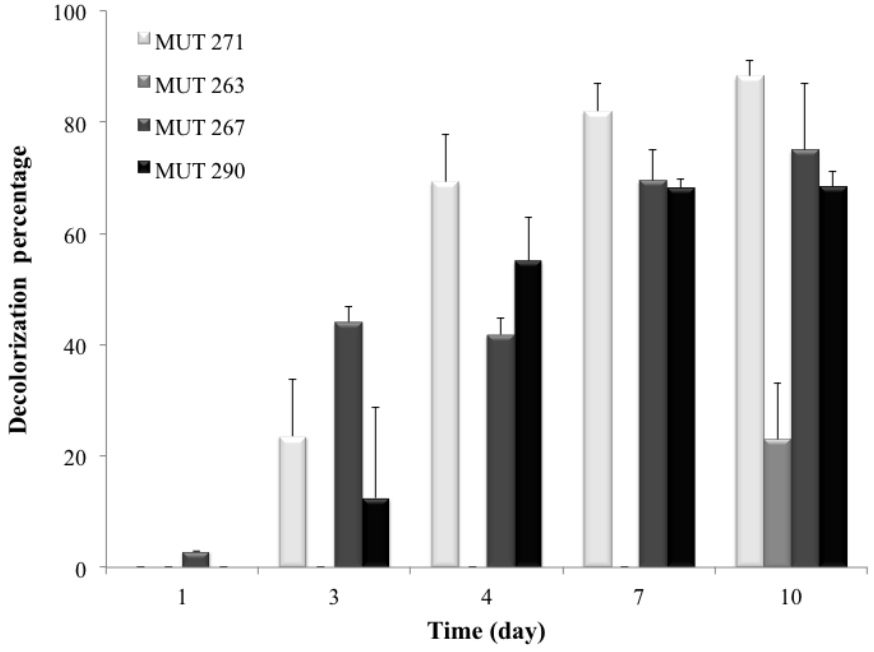

Figure 3. DCPIP decolorization by *A. terreus* MUT 271, Lulworthiales sp. MUT 263, *P. citreonigrum* MUT 267, and *T. harzianum* MUT 290 during time.

The decolorization results are summarized in Table 4 together with the fungal biomasses and the visual disappearance on the crude oil. Data indicated that fungi used the crude oil as source of nourishment: fungal biomasses were significantly higher ($p \leq 0.05$ using Mann-Whitney test) in presence of crude oil (41-53 mg) than in its absence (with ONR7a medium alone below 6 mg, data not shown). The dye displayed a marginal role since in presence of DCPIP alone, fungal biomasses were below 5 mg (data not shown). A correlation between decolorization yields and biomass development could not be drawn. For example, *P. citreonigrum* MUT 267 grew less than *T. harzianum* MUT 290, but they obtained comparable decolorization yields. The capability of fungi to use crude oil was also highlighted by the visual crude oil disappearance that was strongly evident for *A. terreus* MUT 271, followed by *P. citreonigrum* MUT 267 and *T. harzianum* MUT 290.

Table 4. Crude oil degradation assay: DPPIP decolorization percentage, biomass dry weight and visual disappearance of crude oil. Statistical analysis is indicated as letters in parenthesis ($p \leq 0.05$).

Fungal strains	PD (%) ± SD	Dry weight (mg)	Disappearance of crude oil*
A. terreus MUT 271	88.2 ± 2.9 (a)	53.0 ± 7.0 (a)	++
Lulworthiales sp. MUT 263	23.0 ± 10.2 (b)	47.3 ± 12.7 (ab)	-
P. citreonigrum MUT 267	75.0 ± 5.5 (c)	41.0 ± 7.9 (b)	+
T. harzianum MUT 290	68.4 ± 2.8 (c)	51.0 ± 5.2 (a)	+

*Crude oil visual disappearance: - absence; + medium; ++ high.

3.4 Hydrocarbons analysis

Specific removal of petroleum hydrocarbons was determined by analysis of total extracted and resolved hydrocarbons by GC-FID after 10 days of incubation in presence of 1 % w/v of Arabian Light oil added to the fungal pure cultures. The results obtained are summarized in Table 5. Corroborating with DPPIP decolorization tests, *A. terreus* MUT 271 exhibited the highest removal potential of hydrocarbons with a decrease rate of compounds of about 40 % followed by *P. citreonigrum* MUT 267 and Lulworthiales sp. MUT 263 with a rate of decrease of 24 % and 21 %, respectively. According to the GC results, a reduction in oil contamination (< 14 %) was observed also for *T. harzianum* MUT 290 strain. As it illustrated in Figure 4, for all species tested the most conspicuous trend was observed for the higher relative decrease of low-molecular-weight n-alkane

(n-C_{14} to n-C_{18}). This was clearly evident for *P. citreonigrum* MUT 267. Interesting, *A. terreus* MUT 271 showed degradation potential of about 50 % in comparison to the abiotic control for n-C_{14} and n-C_{24} compounds. As a general point of view, all fungi strains exhibited a moderate removal capabilities of petroleum hydrocarbons higher than *n*-C_{30}.

Table 5. Residual percentage of aliphatic hydrocarbons in fungal cultures after 10 days of incubation. Statistical analysis is indicated as letters in parenthesis ($p \leq 0.05$).

Fungal strains	**Aliphatic hydrocarbons (%) ± SD**			
	***n*-C_{14} to *n*-C_{18}**	***n*-C_{24} to *n*-C_{30}**	**> *n*-C_{30}**	**Total**
A. terreus MUT 271	56.7 ± 10.3 (a)	51.0 ± 12.2 (a)	86.7 ± 18.0 (a)	61.8 ± 10.7 (a)
Lulworthiales sp. MUT 263	81.1 ± 4.0 (bc)	82.2 ± 4.1 (b)	90.4 ± 2.2 (b)	78.5 ± 2.3 (b)
P. citreonigrum MUT 267	70.7 ± 7.2 (ba)	84.0 ± 3.4 (b)	84.7 ± 5.6 (b)	75.7 ± 4.2 (b)
T. harzianum MUT 290	84.9 ± 4.6 (c)	85.2 ± 10.5 (b)	90.7 ± 12.0 (b)	85.9 ± 5.9 (b)

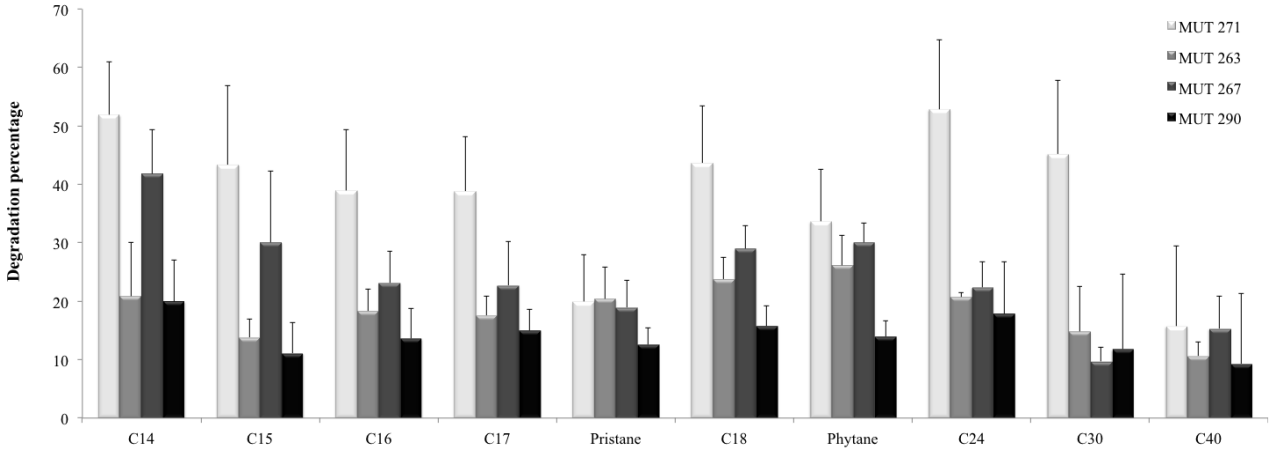

Figure 4. Degradation percentage of principal aliphatic hydrocarbons detected by GC-FID analysis, for the strains *A. terreus* MUT 271, Lulworthiales sp. MUT 263, *P. citreonigrum* MUT 267, and *T. harzianum* MUT 290. Error bar indicates the standard deviation of triplicate measurements.

4 Discussion

Several studies have investigated the presence of bacteria in marine environments contaminated by crude oil (Abbasian et al., 2015; Santisi et al., 2015; Yakimov et al., 2007). However, little is known about the presence and input in biodegradation of fungi in the same contaminated ecosystems. To the best of our knowledge, this is the first complete report on the mycobiota of both water and sediments in a Mediterranean site contaminated by crude oil. The high biodiversity recorded (78

taxa) and the presence of actively growing hyphae in the samples demonstrates the existence of an important living fungal community immediately after the oil spill. These results are in accordance with the reported microbial bloom that usually characterized the oil-polluted environment, immediately after the spill (Bik et al., 2012; Sadaba and Sarinas, 2010). Water and sediments represent very different ecological niches where specific chemical-physicals parameters, including also the bioavailability of oil, affect the microbial community that populate them. This concept was deeply true in the site taken in consideration where the mycobiota recovered from water and sediment display strong differences from both qualitative and quantitative point of view. In particular, the high fungal diversity reported in the water could be related not only to the oil spill but also to the proximity to the coast line, as recently reported by Wang and colleagues (2012) that found a double number of species in seawater near the coast line compared to water far from land or at high depth.

As regard water, the total fungal load (29.7 CFU 100 mL^{-1}) is close to data (18.7-29.6 CFU 100 mL^{-1}) generally reported for uncontaminated marine water (Wang et al., 2012) contrariwise lower than the one reported after an oil spill event in seawater of Philippines (62 x 10^2 CFU 100 mL^{-1}) (Sadaba and Sarina, 2010).

The strong prevalence of Ascomycota is not a surprise and finds many correspondences in literature. Recently, in a review on marine fungi, Jones and collaborators (2015) listed 805, 21 and 3 species of Ascomycota, Basidiomycota and Zygomycota, respectively. Similarly in the crude oil polluted Absheron Penisula of Caspian Sea the most abundant species belong to Ascomycota (Salmanov et al., 2008). In the present study, *Penicillium* and *Aspergillus* represented the most widespread genera followed by *Trichoderma*. *Aspergillus* and *Penicillium* are commonly reported in marine water column (Wang et al., 2012) as well as associated to different substrates. *Trichoderma* appears to be sporadically present in the water column (Saravanan and Sivakumar, 2013) but is commonly associated to marine organisms like algae (Suryanarayanan, 2012). *Aspergillus* (five species) and *Penicillium* (four species) represented also the most widespread genera in the contaminated water of Philippines, where *Trichoderma* was absent (Sadaba and Sarina, 2010). The occurrence of these genera should not surprise because they are well known as degraders of complex organic compounds and endowed of physiological skills that allow them to colonize a marine polluted environment. Indeed *Aspergillus* and *Penicillium* can transform the aliphatic and aromatic crude oil components (Harms et al., 2011) and 11 species of *Trichoderma* were able to grow on different crude oil fraction (Argumedo-Delira et al., 2012).

Among the most abundant water species, *S. racemosum*, *A. tenuissima* and *C. globosum* have already been reported in marine environment (Baakza et al., 2004; Kis-Papo et al., 2003; Sepcic et

al 2011), but this is the first record from oil-contaminated seawater. *S. racemosum* is well known for its ability to oxidize aromatic compounds as benzo[a]pyrene (Cerniglia, 1997). *A. tenuissima* has already been recovered in an oil refinery contaminated soil and was able to use ethanol as sole carbon source (Srivastava et al., 2014). *C. globosum* has been reported in fuel tanks but, its ability to use crude oil was not assessed (Gaylarde et al., 1999).

To the best of our knowledge, among the water isolates, 12 out of 55 taxa (21 %) identified at species level, were reported for the first time in marine environment. Most of them were previously detected in soil (Godinho et al., 2015; Grishkan et al., 2006) and associated to plants (Degenkolb et al., 2006; Zimowska, 2008). All the other species were previously reported as planktonic fungi (Wang et al., 2012), or associated with different kind of marine organisms as *Posidonia oceanica* (Panno et al., 2013), seaweeds (Suryanarayanan, 2012), coral (Raghukumar and Ravindran, 2012) and wood (Garzoli et al., 2015).

Noteworthy from a taxonomic point of view the isolation of several strains of *Corollospora* and Lulworthiales that could represent new phylotypes. *Corollospora* spp. and Lulworthiales are recognized as typical marine fungi (Jones et al, 2009; Kohlmeyer et al., 2000). In detail, the order Lulworthiales contains few genera which display low homologies (< 95 %) with both ITS and LSU available sequences; hence the isolated strains could represent new taxa. As regard *Corollospora*, preliminary phylogenetic analysis (data not shown) on these sterile mycelia show that almost all isolates ascribable to the genus (except one strain identified as *C. portsaidica*) seem to represent a new species. Further analysis will be necessary to clarify the phylogenetic position of these strains.

Sediment sample displayed a total fungal load (about 4,000 CFU g^{-1} dw) higher than that (50-200 CFU g^{-1} dw) reported in chronically contaminated marine sediments in the north of Italy (Salvo et al., 2005), but much lower (2.4 x 10^4 g^{-1} dw) than sites interested by one oil spill event in Philippines (Sadaba and Sarina, 2010). The studied sediments were characterized by a higher biodiversity (17 taxa), compared to the literature: Sadaba and Sarina, (2010) found 9 species in beach soil after an oil spill event while Salvo and colleagues (2005) reported 7 genera. Nevertheless, the sediment biodiversity is lower than those assessed for the surrounding water with a low number of species in common. A possible explanation is in the anoxic condition that usually characterizes crude oil contaminated sediments, where the persistence of the pollution leads to anoxic sediments (Zhu et al., 2004). Hambrich and collaborators (1980) reported a redox potential from + 700 mV to -300 mV for submerged sediments, highlighting that negative values are symptoms of anoxic condition. It is interesting to notice that the redox potential (-140 mV) of the analyzed sediments fit with the anoxic condition reported in literature. Unfortunately, no other data

in literature are available as a comparison between sediments in anoxic condition and fungal community.

As for seawater, the dominance of Ascomycota in contaminated sediments is in agreement with literature data, where Basidiomycota have never been found (Sadaba and Sarina, 2010; Salvo et al., 2005). The highest fungal load in sediments was observed for *C. cladosporioides*: this species, found also in the surrounding polluted water, has been already reported from extreme salinity environments as Saharan salt flat (Jaouani et al., 2014) and in the desert dust (Griffin, 2007). The ability of *C. cladosporioides* to use crude oil in consortium with other organisms has been already signaled (Azarowicz, 1973). Even thought the identification did not reach the species level, Salvo and colleagues (2005) underlined the great diffusion of the genus *Cladosporium* in the highest contaminate sediment, among the studied ones.

P. ambigua is the second widespread species in sediments and, to the best of our knowledge, it is reported for the first time in sandy beaches and in crude oil contaminated environment. Otherwise, this genus was reported in sediments in proximity of mangrove roots (Xu, 2015) and associated to marine algae (Suryanarayanan, 2012).

Noteworthy, among the sediments isolates, 13 out of 14 taxa (92 %) identified at species level were recorded for the first time from this marine environmental matrix. These species usually colonize terrestrial xeric environments as sand dunes (Jones et al., 2011; Mouchacca, 2005) or different kind of soils (Kamolmanit et al., 2013; Rodriguez et al., 1996). Only *P. solitum* was previously found in beach sediments (Gomes et al., 2008).

In order to find out the most promising strains for crude oil bioremediation in marine environment, all the isolated fungi were tested for their capability to grow in presence of crude oil as sole C source. It is interesting to notice that about 1/4 of fungi were stimulated by the presence of crude oil and displayed a vigorous growth respect to controls. Among these fungi, four strains were further studied in liquid condition to analyze their capability to transform crude oil in presence of DCPIP and displays unique features. In detail, *P. citreonigrum* MUT 267 and *T. harzianum* MUT 290 showed a high DP, vigorous growth and a clearly visible crude oil disappearance. Moreover, the petroleum uptake potential of selected strains was confirmed by chemical analysis that showed, at different levels, the removal of a portion of hydrocarbons compounds from liquid pure cultures. In particular *A. terreus* MUT 271 exhibited the best response with the decrease of about 40 % of oil added. This result fit very well with recent papers about the capacity of fungi to degrade oil in the coastal marine environment. Indeed, recently, Al-Nasrawi (2012) reported that three out of the 16 fungi isolated from different localities in Pensacola beach (Gulf of Mexico) which was contaminated with crude oil displayed a high visual discoloration in presence of DCPIP and crude

oil after three days; however only qualitative analyses were done. Simister and collaborators (2015) isolated three fungi (including one strains of *A. terreus*) from oil-soaked sand patties collected from Alabama beaches following the Deepwater Horizon oil spill and demonstrated their strong capability to degrade straight and branches alkanes and alkenes and PAHs. Similarly, Elshafie and colleagues (2007) tested 10 fungal species isolated from tar balls collected from the beaches of Oman for their abilities to grow and degrade n-alkanes and crude oil: different utilization of n-alkanes were underlined, and a strain of *A. terreus* resulted one the best degraders. Hence, *A. terreus* seems to represent a fungal species common in both marine and terrestrial (Ahirwar and Dehariya, 2013; Capotorti et al., 2004; Mohsenzadeh et al., 2012) crude oil contaminated environments endowed of a strong bioremediation potential.

P. citreonigrum has never been reported before from contaminated environments, but often the identification of fungi stops at genus level. Numerous *Penicillium* have been recorded both from marine environment (Saravanan and Sivakumar, 2013) and polluted soils where they have been demonstrated to degrade crude oil (Harms et al., 2011). According to the results obtained when grown in presence of crude oil as sole C source, *P. citreonigrum* MUT 267 showed a high DP, vigorous growth, a clearly visible crude oil disappearance and a moderate removal of aliphatic compounds (24 %). Hence these preliminary results seem encouraging, but further analysis will be performed to confirm the effective degradation activity of this strain.

Different strains of Lulworthiales, isolated from marine environment and exposed to various grade of oil pollution, were able to use crude oil (Kirk and Gordon, 1988). Nevertheless Lulworthiales sp. MUT 263 despite a vigorous growth in presence of crude oil as sole C source showed a moderate DP but was able to remove about 21 % of the total hydrocarbons. Also *T. harzianum* MUT 290 displayed contrasting results in the crude oil degradation test where, despite the high DP and vigorous growth, showed the reduction of about 14 % of the hydrocarbons present in the mixture. *T. harzianum* has been frequently isolated from soils contaminated by crude oil and was demonstrated to reduce about 70 % of DCPIP of napthalene (Ahirwar and Dehariya, 2013) and 26 % of an hydrocarbon mixture in 30 days (Chaıneau et al., 1999). Further investigation will be necessary to better understand the metabolic pathway responsible of crude oil degradation for Lulworthiales sp. MUT 263 and *T. harzianum* MUT 290. The high production of biomass and the contrasting results on the total hydrocarbon removal and/or the DP lead to hypothesize that these fungi could be able to use crude oil fractions not analyzed in the present study. Additional investigations will be necessary to verify the ability of the tested fungi to degrade crude oil in nature, optimizing the degradation efficiency using for example suitable carriers and inocula.

5 Conclusions

Marine crude oil spill is a huge threat for marine ecosystems and wildlife biodiversity and the use of adapted microorganisms able to clean up this mixture of pollutants is an actual challenge for the future. Up to now fungi have been neglected despite their proven degradative abilities. This work provides an overview on the complex mycobiota active in both seawater and sediments of a contaminated site in the Mediterranean area, where a huge fungal biodiversity has been reported with many strains able to use crude oil as the sole C source, in conditions that mimic the natural ones as regards salinity and temperature.

The encouraging results found for *P. citreonigrum* MUT 267 and especially for *A. terreus* MUT 271 in preliminary crude oil degradation experiments clearly show their high potential for bioremediation. Further analyses will be necessary to better characterize their ability to degrade the different fractions of crude oil, in order to set up consortia that can guarantee the maximum effectiveness of bioremediation processes in marine environments.

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